226 HUMAN AMNIOTIC MEMBRANE TO REPAIR OSTEOARTHRITIC CARTILAGE

S.M. Díaz-Prado[1], E. Muiños[2], E. Rendal[3], T. Hermida-Gómez[2], I.M. Fuentes-Boquete[1], M.C. Arufe-Gonda[1], P. Filgueira[4], M.J. Sánchez-Dopico[4], F.J. De Toro[1], F. Galdo[1], F.J. Blanco[1]. *[1]Osteoarticular and Aging Research Laboratory. Biomedical Research Center. CIBER-BBN, Cellular Therapy Area. INIBIC – Hospital Universitario Juan Canalejo. University of A Coruña, Coruña, SPAIN, [2]Osteoarticular and Aging Research Laboratory. Biomedical Research Center. INIBIC-Hospital Universitario Juan Canalejo., Coruña, SPAIN, [3]CIBER-BBN, Cellular Therapy Area., Coruña, SPAIN, [4]Osteoarticular and Aging Research Laboratory. Biomedical Research Center. CIBER-BBN, Cellular Therapy Area. INIBIC – Hospital Universitario Juan Canalejo., Coruña, SPAIN*

Purpose: Human amniotic membrane is a highly abundant and easily accessible tissue that may potentially be an important chondrocyte carrier for cartilage regeneration in vivo.
Objective: Study the feasibility of cryopreserved human amniotic membrane as a chondrocyte carrier for *in vitro* repair human articular cartilage injuries.
Methods: Patches (6×6 cm) of cryopreserved human amniotic membrane were extended into a culture dish. Then human chondrocytes (5×10^5 cells) were cultured over the basal face of the amniotic membrane for 3−4 weeks until 80−90% confluence. These membranes were cut into pieces of 0.7×0.7 cm, and each of them was placed over a chondral defect (6 mm in diameter) of osteoarthritic cartilage with the basal layer facing the defect. The implants were cultured for 16 weeks. Evaluation of *in vitro* chondrocytes culture over the human amniotic membrane and repair, in *in vitro* models, of injured articular cartilage was carried out using histological (hematoxylin-eosin) and immunohistochemical (collagen type I and II) stainings.
Results: Chondrocytes culture over the human amniotic membrane showed that cells grown over the basal layer, but they did not proliferate on the epithelial side. Histological techniques demonstrated that collagen type II but not type I was expressed in the chondrocytes culture on amniotic membrane. Valuation of repair, in 13 *in vitro* repair models, showed the formation of a new tissue on OA cartilage. Integration of new tissue with OA cartilage was excellent. Repair tissue expressed type II collagen in 100% of experiments, however collagen type I was present in 54% of cases.
Conclusions: These results indicated that cryopreserved amniotic membrane can be used as support for chondrocytes proliferation in cell therapy to repair OA cartilage.
Support: Project funded by Servizo Galego de Saúde (PS07/84). Silvia Diaz Prado is beneficiary of an Isidro Parga Pondal contract from Xunta de Galicia, A Coruna, Spain.

227 GLUCOSAMINE PREVENTS AND NF-kB INHIBITORS REDUCE THE CYTOKINE-INDUCED EXPRESSION OF Il-1 IN NORMAL HUMAN ARTICULAR CHONDROCYTES *IN VITRO*

C.H. Lee, M. Glaysher, **H.I. Roach**. *University of Southampton, Southampton, UNITED KINGDOM*

Purpose: Glucosamine (GlcN) supplements are taken by millions of osteoarthritic (OA) patients to ameliorate their symptoms and several large-scale studies have shown a benefit in slowing the progression of the disease. However, the molecular bases for the beneficial effect of GlcN are only beginning to be unravelled. Our aims were to determine whether glucosamine, added to the medium of cultured non-OA chondrocytes, affected the cytokine-induced expression of Il-1β. In addition, we investigated whether the NF-kB pathway is used in the signal transduction mechanisms involved in the cytokine-induced expression of Il-1β.
Methods: Non-OA chondrocytes were isolated by sequential trypsin, hyaluronidase and collagenase digestion from osteoporotic patients who had sustained a fracture of the neck of femur. Previous studies had shown that these chondrocytes are suitable control chondrocytes, because the cells express chondrocytic genes, such as collagens types II, IX and XI, but do not express Il-1β. Cells were maintained in MEM with 5%FCS. Passage 2 cells were divided into the following experimental groups (n = 4 for each group): (I) control culture with no addition; (II) culture with GlcN alone (2 mM); (III) culture with TNF-α/OSM (10 ng/ml and 10 μ/ml) and (IV) culture with TNF-α/OSM in presence of GlcN. In a further series, non-OA chondrocytes were cultured with TNF-α/OSM in presence of two compounds that inhibited the translocation of NF-kB to the nucleus. The first was NF-kB p65 (Ser 276) inhibitory peptide (50 μM) from Imgenex, the second was (E)-2-Fluoro-4'-methoxystilbene (0.2 μM), an analogue of Resveratrol from Merck. Media were changed twice-weekly, when the relevant factors were also added to II, II and IV. Cells were cultured until confluency (2−3 weeks) and semi-quantitative RT-PCR was carried out.
Results: No expression of Il-1β was found in control cultures, with GlcN alone or with the NF-kB inhibitor alone. As expected, TNF-α/OSM induced expression of Il-1β in all cultures. However, the presence of **GlcN completed prevented the cytokine-induced expression of Il-1β**, as indicated by absence of the PCR band. Presence of the NF-kB translocation inhibitors reduced Il-1β expression, but did not abolish it completely, as suggested by reduced intensity of the PCR band.
Conclusions: The present studies demonstrate that glucosamine has the capacity to inhibit the cytokine-induced expression of Il-1β. The glucosamine effect was greater than the effect of interfering with the NF-kB pathway of signal transduction. GlcN is a basic structural unit of chondroitin sulphate and hyaluronan of cartilage proteoglycan aggregates. Hence its beneficial effects were initially suggested to be due to stimulation of GAG synthesis, but the evidence for this mechanism is so far limited. Other studies have shown chondroprotective effects in vitro in that GlcN promoted the chondrogenic phenotype and inhibited MMP-13 expression. Il-1β is thought to play a major role in the progression of osteoarthritis, since Il-1β increases the expression of cartilage-degrading proteases. The present study suggests that GlcN treatment may have beneficial effects through prevention of Il-1β expression, which, in turn, might also reduce expression if its down-stream target genes. However, these are *in vitro* observations and need to be confirmed by *in vivo* studies.

228 THE SYNERGISTIC DRUG COMBINATION CRX-102 INHIBITS MATRIX METALLOPROTEINASE EXPRESSION IN MACROPHAGES AND HUMAN OSTEOARTHRITIC CHONDROCYTES

C.C. Fraser[1], Y. Wang[1], J. Lehar[1], E. Lee[2], K. McCabe[2], S. O'Brien[2], S. Kilfeather[2], A. Borisy[1], **G.R. Zimmermann**[1]. *[1]CombinatoRx Pharmaceuticals, Cambridge, MA, USA, [2]Aeirtec Ltd, Newcastle Upon Tyne, UNITED KINGDOM*

Purpose: Glucocorticoids are therapeutic anti-inflammatory drugs, but are not routinely used to treat osteoarthritis (OA) due to potential side effects. In a proof-of-concept hand OA clinical trial, oral dosing with CRx-102, which is a combination of the cardiovascular drug dipyridamole (Dp) and very low-dose prednisolone (Pd), resulted in a significant reduction in tender and swollen joint counts, suggesting that the combination may modify disease progression. We have previously shown that Dp synergizes with Pd to inhibit inflammation in vitro and in vivo. This synergy allows a much lower dose of Pd to be used to inhibit inflammation, without the side effects associated with glucocorticoids. To determine if Dp and Pd synergize to inhibit matrix metalloproteinase (MMP) expression, we studied LPS-stimulated macrophages and Il-1α stimulated normal human chondrocytes. Additionally, we evaluated the activity of the combination on chondrocytes from human osteoarthritic joints ex vivo. The purpose of this research was to characterize the effect of the combination of Dp and Pd on cells and molecules relevant to the progression of OA.
Methods and Results: Transcriptional profiling (TP) of LPS stimulated macrophages revealed that ADAMTS4 was up-regulated (~20-fold) in response to LPS. Dp (10 uM) in combination with Pd (0.03 uM) inhibited ADAMTS4 expression (67%), more than Dp (47%) or Pd (30%) alone. MMPs 3, 10, 12, 13 and 14 were up-regulated in response to LPS, and like ADAMTS4, were maximally inhibited by the combination of Dp and Pd. Interestingly, analysis of supernatants from stimulated macrophages cultured in serially diluted combinations of Pd and Dp identified strong synergistic inhibition of MMP2 and MMP9. The MAP kinase pathway is thought to play a role in MMP expression. Western blot analysis showed the combination of Pd and Dp inhibited phosphorylation of p38 and JNK but not ERK, while Pd or Dp alone did not. To further characterize the combination on OA-relevant cell types, inhibition of MMPs and other inflammatory mediators by Dp and Pd was measured in Il-1α stimulated primary chondrocytes from normal human donors. Pd demonstrated differential partial inhibition of MMP-3, Il-6, MCP-1 and GRO-alpha. Dp demonstrated complete inhibition of MMP-3 and MCP-1, partial inhibition of GRO-alpha, and no inhibition of Il-6. OA chondrocytes were isolated from osteoarthritic joint cartilage obtained from total knee replacement surgery, and the ability of Dp and Pd to suppress Il-1 induced production of pro-inflammatory mediators including MMPs was assessed. Dp and Pd alone and in combination suppressed production of TNFα, MIP-1α, MIP-1β, MCP-1, RANTES, and MMP-13.
Conclusions: We show here that Pd and Dp synergize to inhibit MMP production in macrophages and decrease expression in human

osteoarthritis-derived chondrocytes ex vivo. The synergy between Pd and Dp allows a very low dose of Pd to be used clinically, potentially overcoming many glucocorticoid-related side effects. The combination (CRx-102) has demonstrated efficacy in a Phase 2a hand OA trial and in a Phase 2a rheumatoid arthritis trial. The broad anti-inflammatory synergy of Dp and Pd, and the ability to inhibit MMPs may contribute to clinical efficacy, supporting the notion that the sensitization to glucocorticoids by dipyridamole may alter disease progression in OA.

229 UP-REGULATED EXPRESSION OF ANK IN JOINT TISSUE FROM PATIENTS WITH CALCIUM PYROPHOSPHATE DIHYDRATE CRYSTAL DEPOSITION DISEASE (CPPD)

M. Uzuki[1], L.M. Ryan[2], T. Sawai[1], I. Masuda[2]. [1]*Iwate Medical University, School of Medicine, Morioka, Iwate, JAPAN,* [2]*Medical College of Wisconsin, Milwaukee, WI, USA*

Purpose: Extracellular inorganic pyrophosphate (ePPi) promotes CPPD crystal formation in cartilage matrix in patients with osteoarthritis. ANK was recently proposed to be an important factor in transport of intracellular inorganic pyrophosphate (iPPi) across the cell membrane. The ANK protein is a multiple transmembrane protein either serving as an anion channel or as a regulator of such a channel. In this study, we examined the expression of ANK in the joint tissue of patients with CPPD to study its possible biological roles in the manifestation of this disorder.
Methods: Cartilage, meniscus and synovial tissues used in this study were obtained from 15 patients with osteoarthritis with CPPD crystals (CPPD), 10 patients with osteoarthritis without crystal (OA) and 2 normal from autopsy cases. We studied the expression of ANK in joint tissue using immunohistochemistory. As a negative control, non-immune rabbit IgG was used instead of primary antibody for ANK. We counted the number of ANK positive cells in the joint tissues.
Results: ANK immunoreactivity was detected in synovial cells, chondrocytes and matrix of cartilage and meniscus. The numbers of ANK positive cells in the joint tissues was significantly higher in CPPD than in OA or normal (Fig. 1: cartilage, Fig. 2: meniscus, Fig. 3: synovium). The immunoreactivities reached maximum levels in hypertrophic chondrocytes around the deposits of crystals, both in their number and intensity, especially in their cell membranes.
Conclusions: ANK was seen in the joint tissue of patients with CPPD, especially around the deposits of crystals. These findings suggest that up-regulated ANK experession contributes to CPPD crystal forming cartilage. ANK may control iPPi egress across the cell membrane.

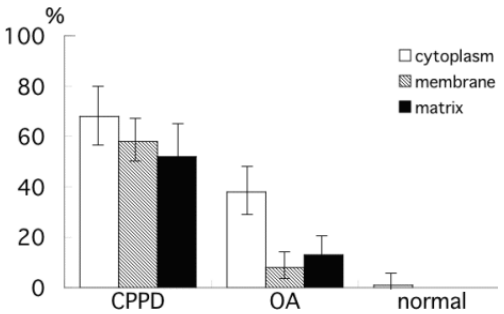

Figure 1.

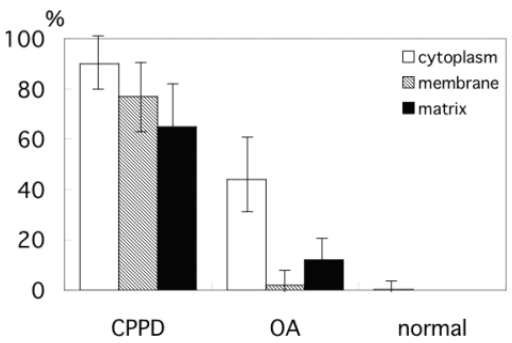

Figure 2.

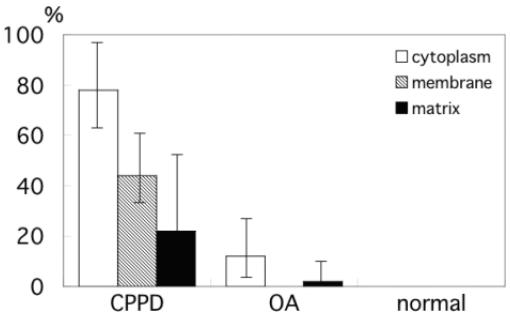

Figure 3.

230 LEPTIN ENHANCES MMP-1, MMP-3 AND MMP-13 PRODUCTION IN HUMAN OA CARTILAGE

K. Vuolteenaho[1], A. Koskinen[1], R. Nieminen[1], T. Moilanen[2], E. Moilanen[1]. [1]*The Immunopharmacology Research Group, Medical School, University of Tampere and Tampere University Hospital, Tampere, FINLAND,* [2]*Coxa Hospital for Joint Replacement and The Immunopharmacology Research Group, Medical School, University of Tampere, Tampere, FINLAND*

Purpose: Obesity is an important risk factor for OA of weight-bearing joints, but also for hand joints, pointing to an obesity-related metabolic factor that influences on the susceptibility or pathogenesis of OA. Leptin is a proinflammatory adipokine regulating energy balance and it has recently been related also to arthritis and cartilage metabolism. In OA, proteolytic degradation of cartilage is mediated by matrix metalloproteinases. In the present study, the effects of leptin on MMP-1, MMP-3, MMP-8 and MMP-13 production in human OA cartilage were studied.
Methods: Cartilage tissue obtained from the leftover pieces of total knee replacement surgery from patients with OA was used in the experiments. MMP production in the culture medium was measured by multiplex bead array method (Luminex).
Results: Leptin alone and in combination with Il-1 enhanced production of collagenases MMP-1 and MMP-13, and stromelysin-1 (MMP-3) in human OA cartilage, while collagenase-2 (MMP-8) concentrations remained undetectable. The effects of leptin on MMP-1, MMP-3 and MMP-13 production were mediated through transcription factor NF-κB, and through PKC and MAP kinase JNK. In addition, p38 pathway was involved in the leptin-induced MMP-1 and MMP-13 production and Erk1/2 pathway in MMP-1 production.
Conclusions: The findings support the idea of leptin as a detrimental factor in OA cartilage and as a link between obesity and increased risk for osteoarthritis.

231 ROLE OF SP1 TRANSCRIPTION FACTOR IN INTERLEUKIN-1 INDUCED ADAMTS-4 GENE EXPRESSION IN HUMAN ARTICULAR CHONDROCYTES

J. Sylvester, R. Ahmad, **M. Zafarullah**. *University of Montreal, Montreal, QC, CANADA*

Purpose: Proinflammatory cytokines such as interleukin-1 beta (Il-1b) stimulate cartilage extracellular matrix aggrecan degradation by aggrecanases or ADAMTS (a disintegrin and metalloproteinase with thrombospondin-like motif). Human aggrecanase-1 (ADAMTS-4) gene promoter contains one specificity protein-1 (Sp1)-transcription factor-binding site. We investigated the previously unknown role of Sp1 in the regulation of ADAMTS-4 gene expression.
Methods: Normal human knee chondrocytes were grown in Differentiation Bullekit medium as high-density short-term monolayer cultures. Cells were either treated with various pharmacological inhibitors or transfected with senase and antisense Sp1 oligonucleotides as well as negative control or Sp1-specific siRNAs. Subsequently, cells were stimulated with Il-1β. Knockdown of respective proteins and effect on aggrecanase-1 was studied by RT-PCR and Western blot analysis.
Results: Mithramycin and WP631, the specific inhibitors of guanine cytosine-rich SP1 DNA binding partially suppressed Il-1-induced expression of ADAMTS-4. Genetic inhibition of Sp1 by transfection of antisense oligonucleotide or Sp1 knockdown by RNA interference with small interfering RNA (siRNA) partially inhibited ADAMTS-4 induction by Il-1. Sense oligonucleotide and negative control siRNA had no effect.